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Arbuscular mycorrhizae and dark-septate endophytes on *Atriplex cordobensis* in saline sites from Argentina

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Atriplex cordobensis occurs in salt works and saline soils of northern and central Argentina and is utilized as a forage source; however the mycorrhizal status of this salt bush is unknown. We studied the arbuscular mycorrhizae fungi (AMF) and dark-septate endophytes (DSE) fungi in *A. cordobensis* analysing infection of the roots and spores in the rhizopheric soil. Soil and root samples were collected during summer 2008 at three different saline sites (Salinas Grandes de Jujuy (SGJ), Quebrada de Cafayate-Salta (QCS) and Salinas Grandes de Córdoba (SGC). Assessment of AMF and DSE colonization were made by washing and staining the roots and the AMF spores number was estimated by standard sieving and decanting methods. The results showed moderate to high levels of AMF colonization as well as significant differences in AM colonization and AMF spores number between sites. *Glomus s.l.* was the dominant genus in *A. cordobensis* rhizosphere. DSE colonization did not show significant differences between sites. This study confirmed the occurrence of AMF and DSE in *Atriplex cordobensis* in stressed environments of Argentina.

Key words: AMF and DSE correlation · root colonization · saline soils ecology· spore number.

Introduction

About 75% of the Argentina´s land comprises arid and semiarid regions in which the soil salinization process is a serious problem that increases steadily (Giri *et al*., 2003; Al- Karaki, 2006). Under these conditions, halophyte plants are known to tolerate high levels of salinity and can reach optimal levels of growth under saline conditions (Flowers *et al.,* 1977; Ungar, 1991). The most characteristic plant community in these saline habitats is the halophytic vegetation or "jumeal" composed of several Chenopodiaceae species, where *Atriplex cordobensis* is one of the most abundant.

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The role of arbuscular mycorrhizal fungi (AMF) in vegetation stress alleviation is well known (Hildebrandt *et al.,* 2001; Plenchette and Duponnois, 2005). Colonization of AMF has been reported in *Atriplex* spp. and *Salsola* spp. under drought and/or salt-stress conditions (Hirrel *et al.,* 1978; Kim and Weber, 1985; Katembe *et al.,* 1998), despite they are generally regarded as non-mycorrhizal. In particular several species of *Atriplex (A. nummularia, A. canescens, A. confertifolia, A. gardneri, A. polycarpa, A. versicaria, A. spinosa, A. lampa* and *A. argentina*) have shown AMF association (Allen, 1983; Allen and Allen, 1990; Plenchette and Duponnois, 2005; Cofré *et al.*, 2007; Soteras *et al.*, 2009), however, the mycorrhizal status of *A. cordobensis* is currently unknown. In the field, native populations of *Atriplex* spp. have been found to be more often colonized by dark septate endophytic (DSE) fungi than by AMF (Barrow and Osuna, 2002). DSE comprises a group of ascomycetous fungi that colonize roots of many plants, both intra-and intercellularly (Jumpponen, 2001) that range from mutualism to parasitism (Jumpponen, 2001; Mandyam and Jumpponen, 2005) but its role within the roots still remains unresolved (Smith and Read, 2008). However, their widespread occurrence in cold or drought-stressed ecosystems, their potential to function as mycorrhizal fungi and the extensive internal colonization by active structures suggest that these endophytes are significant components of stressed ecosystems (Haselwandter and Read, 1982; Jumpponen, 2001; Barrow and Osuna, 2002).

The use of halophytic plants in fodder production constitutes a useful practice in order to restore the vegetation of salt-affected areas (Yeo and Flowers, 1980; O`Leary, 1988). In this sense, *A. cordobensis* is currently used as source of forage in saline areas of northern Argentina (Salem *et al.,* 2010) and its symbioses with AMF would be important for their growth and establishment. In the present study, we examined the presence of AMF and DSE in *A. cordobensis* and evaluated root colonization and AMF spore taxa at three different saline sites in Argentina.

Materials and methods

Sampling sites

Samples were obtained in three locations in Northwestern Argentina, namely: 1) Salinas Grandes, Córdoba province (SGC), 29° 44’ 12.5” S and 64° 31’ 46.4” W, 177 masl, characterized by a Typical Salortide soil type, 500 mm of annual precipitation and a mean annual temperature of 19.9 ºC. 2) Quebrada de Cafayate, Salta province (QCS), 26° 03’ 11.8” S and 65° 52’ 59.6” W, with an elevation of 1557 masl average, a Typical Halacueptes soil type, annual precipitation 375 mm and a mean annual temperature 14.8ºC. 3) Salinas

Grandes, Jujuy province (SGJ), 23° 37' 36.43'' S and 65° 51' 06.02" W, 3417 masl; the soil is classified as Typical Torrifluventes, average annual precipitation 300 mm and a mean annual temperature of 8 ºC. The sites are characterized by a medium shrub steppe (average coverage: 20%) in which shrubs are dispersed and mostly growth in isolated groups, with a relatively developed herbaceous and pastures layer (average coverage of herbs and pastures: 15%).

The plant community surrounding the saline sites 1 (SGC) and 2 (QCS), usually out of the reach of saline conditions is dominated by trees such as *Aspidosperma quebracho blanco* Schltdl., *Prosopis flexuosa* DC., *Cercidium australe* IM Johnst., *Mymozyganthus carinatus* (Grisseb.) Bukart, *Ziziphus mistol* Griseb., *Prosopis torquata* (Cav. ex Lag.) DC., *Stetsonia coryne* (Salm-Dyck) Britton and Rose and *Larrea divaricata* Cav. Within the sites the vegetation is characterized by small shrubs such as *Allenrolfea patagonica* (Moq.) Kuntze, *Heterostachys ritteriana* (Moq.) Moq., *Atriplex argentina* Speg., *A. lampa* (Moq.) D. Dietr. *Salicornia ambigua*, *Suaeda divaricata* Moq (Cabido and Zak, 1999).

The vegetation of site 3 (SGJ) is characterized by nanophanerophytes usually spiny, with resins, leaves leathery, hairy, small or reduced to thorns. The dominant species are *Lycium chilense* Miers ex Bertero, *Adesmia horrida* Gillies ex Hook & Arn., *A. pinifolia* Gillies ex Hook & Arn., *Senecio oreophyton* J. Remy, *Fabiana denudata* Miers, *Stipa speciosa* Trin. & Rupr., *Maihueniopsis glomerata* (Haw.) R. Kiesling, *Artemisia mendozana* DC., *Parastrephia quadrangularis* (Meyen) Cabrera, *Atriplex* spp., *Verbena* spp. (Cajal *et al.*, 1998).

During January 2008 (summer) whole root system of the plants together with the rhizospheric soil (soil adjacent to the root system) of six isolated plants of *A. cordobensis* were collected in each of the three sites. These soil samples were collected with a metal corer (10 cm of diameter) to a depth 0-15 cm. Each sample consisted of 20 sub-samples that were pooled and mixed to obtain one composite sample (700 g). The samples were placed in plastic bags and stored at 4 ºC during transport to the laboratory. From these soil samples we extracted the AMF spores.

Soil analysis

To characterize the soil in each site, a fraction of the soil core from each plant was separated and pooled in one soil sample per site. Soil samples were air-dried and sieved (2 mm mesh) and the ≤ 2 mm fraction was analyzed as follows. Soil pH was determined in 1:1, soil: water; electrical conductivity of a saturation extract was measured at 25 ºC (Bower and Wilcox, 1965). Available

phosphorus was determined following Bray and Kurtz I (Jackson, 1964). Organic matter content was determined following Nelson and Sommers (1982). Total nitrogen was determined using the micro-Kjeldhal method (Bremner and Mulvaney, 1982). Exchangeable sodium percentage in saturated soil extracts, calcium carbonate equivalent and soil texture were also determined. The physicochemical soil features of the three sites are shown in Table 1.

Atriplex cordobensis root colonization

Roots were separated from the soil core and washed with tap water to remove soil particles and debris. Roots were cleared in 10% KOH (15 min at 90°C), acidified with 1% HCl (1 min, room temperature) and stained in 0.05% trypan blue (5 min, 90°C) (Phillips and Hayman, 1970). Roots that remained dark after clearing were bleached with 30% H_2O_2 before staining. For observation of DSE, cleared roots were observed directly. Multiple root segments (25-30) of 1 cm long from each plant were mounted on glass slides in PVLG (polyvinyl alcohol-lactic acid-glycerin) and viewed under a compound microscope at 40x magnification (McGonigle *et al.,* 1990). The presence of AMF or DSE structures was scored for 100 intersections of root and reticule line per plant. An intersection was considered colonized if the reticule intersected vesicles, hyphal coils, non-septate hyphae, DSE-melanized septate hyphae, and microsclerotia.

Arbuscular mycorrhizal fungi

Spores and sporocarps were extracted from the soil samples by wet sieving and decanting (Gerdemann and Nicolson, 1963), followed by centrifugation in sucrose gradient (Walker *et al.,* 1982). Only apparently healthy spores were counted in a 9 cm Petri dish by direct observation under a binocular stereomicroscope. For the morphospecies analysis, fungal spores and sporocarps were mounted into slides in PVLG with and without the addition of Melzer´s reagent (Omar *et al.,* 1979). Taxonomic identification of these structures was achieved by following keys of Schenck and Perez (1990) and INVAM (http://www.invam.caf.wvu.edu). Spores were identified up to the genus or when was possible to species level. Spore number was expressed as number of AMF spores in 100 g of dried soil.

Statistical analysis

The data distribution of AMF colonization, DSE colonization and AMF spore number was not normal (Kolmogorov-Smirnov and Shapiro-Wilks

normality tests), and variances were not homogeneously distributed (Levene Test). The average of AMF and DSE colonization and AMF spore number were analysed using Kruskall-Wallis. The relationships among AMF colonization, AMF spore number and DSE colonization, and the three sites were analyzed by Spearman correlations. Analysis was conducted using InfoStat v. 1.0 (Di Rienzo *et al.,* 2002).

Results

Soil characterization

Soils were slightly alkaline with high electrical conductivity. They differed in texture and nutrient content. Salinas Grandes de Jujuy presented low soil concentrations of available phosphate and a higher % of organic matter, electrical conductivity and the ions that raise the EC value (Ca^{++}, Na^{+} and Cl^{-}) in comparison with the other two sites. In Quebrada de Cafayate, soils presented higher P levels than the other two sites. This soil showed intermediate levels of organic matter, total N, and field capacity and the lowest value of EC. Salinas Grandes de Córdoba presented the lowest values of Cox (oxidable carbon), organic matter and field capacity, and intermediate values of pH, P and electrical conductivity (Table 1).

Table 1. Physicochemical soil features of the three sites, Salinas Grandes de Córdoba (SGC), Quebrada de Cafayate-Salta (QCS) and Salinas Grandes de Jujuy (SGJ) as analized from soil profiles taken during field work

Parameters	**SGC**	**QCS**	**SGJ**
Texture	Loam sandy	Sandy loam	Loam silty
Cox. (%)	0.9	1.5	1.2
OM (%)	1.1	1.5	2.0
pH	8.2	8.6	7.9
EC (mS/cm at 25°C)	4.42	2.09	19.77
P (ppm)	22.23	44.8	5.4
N (%)	0.07	0.07	0.10
FC (% of dry weigh)	12.7	27.1	30.0
Ca^{++}	39	18.5	322.7
Mg^{++}	9.2	3.7	154.7
Na^{+}	303.8	72.8	936
K^{+}	2	7.3	8.8
HCO_3^{-}	11.2	8	12.9
SO_4^{2-}	99.8	17.2	40.5
Cl^{-}	261.9	85.8	1447.4
ESP (%)	23.8	9.3	23.3

$C_{ox.}$: oxidable carbon, OM: organic matter, EC: electrical conductivity of extract at 1:2.5 water dilution, P: phosphorus, N: nitrogen, FC: field capacity, ESP: exchangeable sodium percentage

AMF root colonization

Atriplex cordobensis presented abundant AMF structures in their roots. This saltbush showed a colonization type characterized by inter- and intracellular branched hyphae of 3.22-14.6 µm in diameter and oval and circular smooth walled vesicles (28.4-77.1 µm in diam.) with smooth walls and subtending hyphae of 3-7 µm of diameter. (Fig. 1A-E). Hyphal coils (Fig 1D) were present and restricted to few outer cells of the cortical root close by the entry points. Arbuscules were not observed within the root segments of the *A. cordobensis* analyzed.

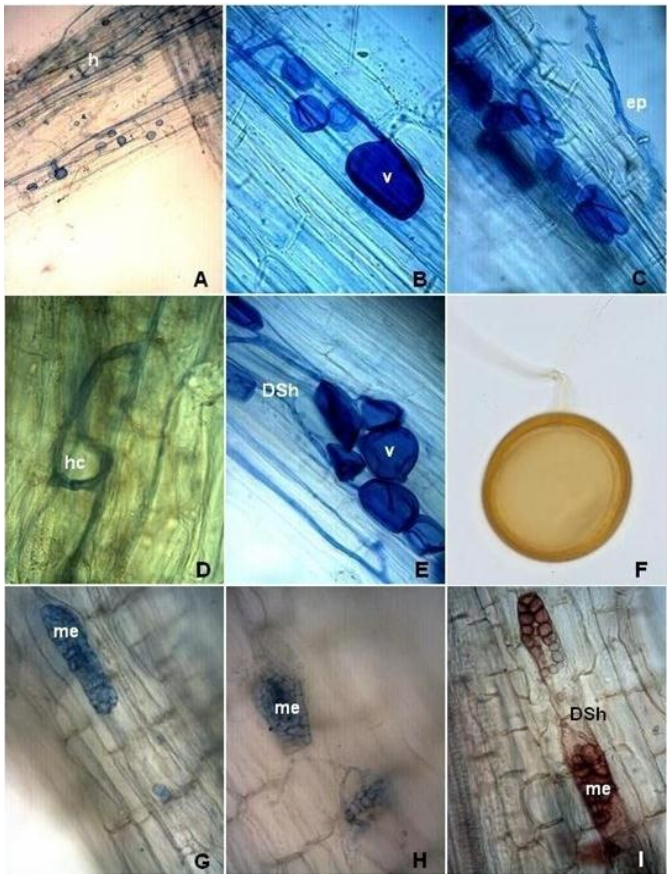

Fig. 1. AMF and DSE in *Atriplex cordobensis*. A-E: hyphae (h), vesicles (v), entry point (ep), hyphal coil (hc), DSh (Dark Septate hypahe). F: *Funneliformis geosporum*. G-I: DSh (Dark Septate hyphae) microesclerotia (me). Scale bar A-I 50 µm

The percentage of AMF colonization (% AMF colonization) ranged from 0 to 99%. AMF colonization differed significantly between sites (H=13.05,

p<0.001) (Fig. 2), been lower in Salinas Grandes de Jujuy (SGJ) than in Quebrada de Cafayate (Salta) (QCS) and Salinas Grandes de Córdoba (SGC). The mean level of AMF colonization for each site was: 59.95 % (SE: 12.07) in SGC (range 18 to 99.17%); 25.39 % (SE: 5.09) in QCS (range 12.67 to 41.33 %) and 2.64 % (SE: 1.34) in SGJ (range 0 to 8.83 %) (Fig. 2).

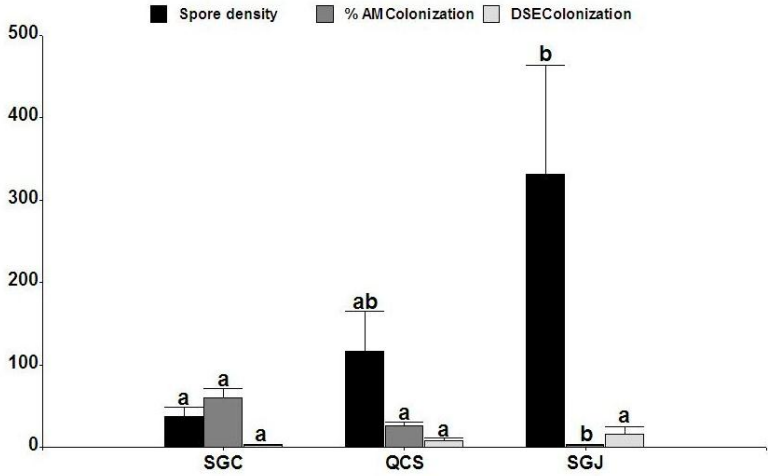

Fig. 2. Spore density (spores number in 100 g of dry soil), arbuscular mycorrhizal colonization (%) and dark septate endophytes colonization (%) present in *Atriplex cordobensis* in rhizospheric soil and roots from Salinas Grandes, Córdoba province (SGC), Quebrada de Cafayate, Salta province (QCS) and Salinas Grandes, Jujuy province (SGJ). Bars with the same letters are not significantly different between sites (Tukey test, P<0.05)

The percentage of vesicles differed significantly between sites (H=9.91, *p*<0.001). For SGC the percentage of vesicles was 14 %, for QCS 2.5 % and for SGJ 0.49%.

AMF spore number

AMF spore number ranged from 8.7 to 969.5 spores per 100 g. soil. There were significant differences between AMF spore number and sites (H=7.82, *p*<0.05). Average spore number was significantly higher in SGJ than in SGC (Fig.2). The genera *"Glomus* sensu lato*"* and *Acaulospora* were identified, presenting an average of 160.09 (standard error (SE): 53.20) and 1.13 (SE: 0.63) spores per 100 g soil respectively. *"Glomus* sensu lato*"* was the dominant genus.

The dominant species of AMF spores associated to *A. cordobensis* were *Archaeospora* aff. *leptoticha* (N.C. Schenck & G.S. Sm) J.B. Morton &

D.Redecker, *Acaulospora scrobiculata* Trappe, *A. bireticulata* F.M. Rothwell & Trappe, *Funneliformis geosporum* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler, *Rhizophagus clarus* (T.H. Nicolson & N.C. Schenck) C. Walker & A. Schüßler, *Glomus brohultii* R.A. Herrera, Ferre & Sieverd., *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüßler, *Claroideoglomus luteum* (L.J. Kenn., J.C. Stutz & J.B. Morton) C. Walker & A. Schüßler and *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler. *Archaeospora* aff. *leptoticha* spores were presented only in one plant from Salinas Grandes de Jujuy, and this record was not included in the statistical analysis. The most frequent and abundant species in all three sites was *F. geosporum* (Fig. 1F).

DSE colonization

DSE were frequently observed along with AMF colonization in *A. cordobensis* roots. Sparsely colonizing hyphae (Fig 1 E, I) and microsclerotia (Fig. 1G-I) were recorded in some roots segments. DSE colonization was characterized by parallel light brown to black runner hyphae 2-4 µm wide. Intraradical colonization was regarded as by frequently septate intercellular melanized hyphae and intracellular microsclerotia formation (mass of densely packed, dark hyphae filling epidermal and exodermis cells). DSE colonization ranged between 0-33%, although there were no significant differences between sites (Fig. 2). The mean level of DSE colonization for each site was: 1.25 % (SE: 0.24) in SGC; 6.75 % (SE.1.26) in QCS and 12.08 % (SE: 6.74) in SGJ (Fig. 2).

AMF-DSE colonization and AMF spore number correlations

Significant negative correlations occurred between percentage of AMF colonization and sites (r=- 0.7621, p<0.01) as well as spore number and % of AMF colonization (r= -0.6223, p<0.05). A positive correlation was observed between spore number and sites (r=0.6858, p<0.01). No relationship was found between AM fungi and DSE colonization (r= 0.134, p= 0.58), nor between vesicles and microsclerotia (r= 0.008, p= 0.974).

Discussion

The present study reports and illustrates mycorrhizal symbionts associated to *A. cordobensis* in three different saline sites in Argentina. The study of saline soils showed similar values of exchangeable sodium percentage, organic matter and pH than those found by other authors for arid environments

(Aliasgharzadeh *et al.*, 2001; Landwehr *et al.*, 2002). Despite the observation of mycorrhizal structures such as inter- and intracellular vesicles and hyphae within the roots of *A. cordobensis* an AM morphological type of colonization cannot be clearly defined, because of the absence of arbuscules and scarcity of hyphal coils. Certainly, Plenchette and Duponnois (2005) hypothesized about the existence of a third AM morphological type with no arbuscules in the Chenopodiaceae family. However, the short longevity of the arbuscules or their formation in a specific periods of the year (based on seasonal effects or environmental stress) may cause their absence (Brundrett and Kendrick, 1990a; Yawney and Schultz, 1990; Mullen and Schmidt, 1993).

The abundance of ellipsoid intraradical vesicles in *A. cordobensis* roots, suggested the presence of species within the order Glomerales (Schüßler *et al.*, 2001). AMF belonging to the genus *"Glomus* sensu lato*"* were observed to be dominant in the rhizosphere soil of *A. cordobensis* at all sites, as previously observed (Cordoba *et al.*, 2001, Wang *et al.*, 2004, Yamato *et al.*, 2009 and Boonlue *et al.*, 2012). Within this group, *Funneliformis geosporum* was the most frequent and abundant species in our sites, similarly in previous studies by Carvalho *et al.* (2001); Hildebrandt *et al.* (2001); Landwehr *et al.* (2002) and Wilde *et al.* (2009), found this species to be dominant in saline-alkaline soils from Europe.

The percentage of AMF colonization observed in *A. cordobensis* in Argentina, was significantly higher (up to 90%) (average 29.32 %) when compared with *Atriplex argentina* from Salinas Grandes de Córdoba (18 %) (Cofré *et al.*, 2007), and also than the registered for other halophytes from saline sites (0.7 to 5.2 %) described elsewhere in similarly arid ecosystems (Landwehr *et al.*, 2002; Wang *et al.*, 2004; Lugo *et al.*, 2005, Tian *et al.*, 2006) Nutrient status and salinity have been reported to decrease AMF root colonization and spore number in soil (Escudero and Mendoza, 2005). In this study, the SGJ site presented the lowest AMF colonization and the highest AMF spore number compared with the other two sites (QCS and SGC). The last site presented the highest values of AM colonization and % of vesicles and the lowest AMF spore number. This trend was supported by correlation analysis, where a negative correlation was observed between the percentage of AMF colonization and sites; and a positive correlation between AMF spore number and sites. Although, exchangeable sodium percentage between the sites SGC and SGJ is about the same, other ions raising the EC value such as sodium, chloride and calcium could influence the AMF colonization (Juniper and Abbott, 2006; Sheng *et al.*, 2008). Besides, the content of phosphorus is the lowest at the SGJ site which could also affect AMF colonization. It is known that many environmental factor, such as EC and P values observed in this

study, could affect the physiology of the host plant and its fungal symbionts (Evelin *et al.,* 2009), however it is evident that other biotic and abiotic factors might be affecting the spore number and root colonization of AMF in natural conditions, such as altitude in this case, but to estimate their influence is far beyond the aim of this study.

The abundance of fungal spores found at the SGJ might be resulted to the saline stress that stimulates sporulation (Tressner and Hayes, 1971), as well as the extreme temperatures and light intensity that characterizes high plateaus. It is known that AM fungi may produce spores at low root-colonization levels in severe saline conditions (Aliasgharzadeh *et al*., 2001). Our data indicated a negative correlation between spore number and % AMF colonization, in contrast to other reports on saline soils where low or even zero spore number was found (Hirrel *et al*., 1978; Kim and Weber, 1985; Barrow and Osuna, 2002). *Atriplex cordobensis* showed DSE in their roots being the first report of this fungal association. In accordance to other studies, intracellular mycelium and microsclerotia were also reported for *Atriplex* species, such as *A. canescens* (Barrow and Aaltonen, 2001, Barrow and Osuna, 2002) other Chenopodiaceae hosts (Urcelay *et al.,* 2010), and plants from other stressed environments (Read and Haselwandter, 1981; Newsham, 1999, 2009; Bagyalakshmi *et al.,* 2010).

Despite the fact we have not been able to cultivate the DSE in vitro, the morphological constitution of the microsclerotia and type of colonization are similar to those described by Yu *et al.* (2001) and Becerra *et al.* (2009) for *Phialocephala fortinii*.

Although, in this study we did not observed significant differences between DSE colonization and sites, the raw results show that DSE colonization increase at SGJ. AMF colonization was not correlated with DSE colonization. Despite that DSE differ in strategy (Haselwandter and Read, 1980; Treu *et al.,* 1996) and morphology compared with AM fungi, their function might be similar in response to the harsh conditions of dry soils (Barrow and Aaltonen, 2001). DSE are found extensively in nutrient-stressed environments where AM fungi generally do not proliferate (Kohn and Stasovski, 1990).

The lack of correlation between DSE and AMF suggest independence between the two symbiotic relationships and the absence of interference or physiological complementarities between them as observed in other studies such as Haselwandter and Read (1980); Treu *et al.* (1996). Further studies are necessary to elucidate the ecological role of AM fungi occurring in *Atriplex cordobensis* and other members of the Chenopodiaceae in stressed environments such as the saline sites of Argentina.

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